Transient Overexpression of -Ca^{2+}/Calmodulin-Dependent Protein Kinase II in the Nucleus Accumbens Shell Enhances Behavioral Responding to Amphetamine

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Transient Overexpression of α-Ca^{2+}/Calmodulin-Dependent Protein Kinase II in the Nucleus Accumbens Shell Enhances Behavioral Responding to Amphetamine

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Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is known to contribute to the expression of psychostimulant sensitization by regulating dopamine (DA) overflow from DA neuron terminals in the nucleus accumbens (NAcc). The present experiments explored the contribution of CaMKII in NAcc neurons postsynaptic to these terminals where it is known to participate in a number of signaling pathways that regulate responding to psychostimulant drugs. Exposure to amphetamine transiently increased αCaMKII levels in the shell but not the core of the NAcc. Thus, HSV (herpes simplex viral) vectors were used to transiently overexpress αCaMKII in NAcc neurons in drug-naive rats, and behavioral responding to amphetamine was assessed. Transiently overexpressing αCaMKII in the NAcc shell led to long-lasting enhancement of amphetamine-induced locomotion and self-administration manifested when αCaMKII levels were elevated and persisting long after they had returned to baseline. Enhanced locomotion was not observed after infection in the NAcc core or sites adjacent to the NAcc. Transient elevation of NAcc shell αCaMKII levels also enhanced locomotor responding to NAcc AMPA and increased phosphorylation levels of GluR1 (Ser831), a CaMKII site, both soon and long after infection. Similar increases in pGluR1 (Ser831) were observed both soon and long after exposure to amphetamine. These results indicate that the transient increase in αCaMKII observed in neurons of the NAcc shell after viral-mediated gene transfer and likely exposure to amphetamine leads to neuroadaptations in AMPA receptor signaling in this site that may contribute to the long-lasting maintenance of behavioral and incentive sensitization by psychostimulant drugs like amphetamine.

Introduction

Exposing animals to amphetamine enhances the ability of this drug to produce locomotor activation, increase nucleus accumbens (NAcc) dopamine (DA) overflow, and support drug taking (Kalivas and Stewart, 1991; Paulson and Robinson, 1995; Vezina et al., 2002). These phenomena, manifestations of drug sensitization, may model the transition from casual drug use to craving and abuse (Robinson and Berridge, 1993; Vezina, 2004). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a serine/threonine kinase that phosphorylates a wide array of downstream targets and is highly expressed in forebrain sites like the NAcc (Goto et al., 1994; Solà et al., 1999), contributes to both the induction and expression of sensitization by psychostimulants. Thus, CaMKII inhibitors prevent initiation of the neuroadaptations in midbrain leading to sensitized responding (Licata and Pierce, 2003) and, when applied to DA terminal regions in NAcc and striatum, prevent the expression of established sensitization, including enhanced locomotion (Pierce et al., 1998), DA release (Pierce and Kalivas, 1997; Kantor et al., 1999), and drug taking (Loweth et al., 2008).

Although the evidence that CaMKII mediates the expression of psychostimulant sensitization suggests that it interacts with substrates in DA neuron terminals in forebrain to regulate enhanced DA release, additional evidence indicates that CaMKII can also act postsynaptically to regulate a number of signaling pathways known to mediate enhanced responding to psychostimulants. CaMKII can inhibit the activity of cAMP-response-element-binding protein (CREB) (Wu and McMurray, 2001), a transcription factor activated by stimulants in striatal neurons (Cole et al., 1995; Turgeon et al., 1997), by phosphorylating it at serine residue 142. Overexpressing a dominant-negative form of this protein in NAcc neurons enhances the rewarding properties of cocaine (Carlezon et al., 1998; Pliakas et al., 2001), suggesting that NAcc CaMKII could enhance drug responding possibly by inhibiting CREB activity. CaMKII also directly phosphorylates the AMPA receptor subunit GluR1 at serine residue 831 to enhance channel conductance (Song and Huganir, 2002) and is required for GluR1 insertion into the synapse (Hayashi et al.,

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2000). A number of molecular as well as AMPA receptor blockade and activation studies indicate that glutamatergic transmission plays an important role in the expression of sensitization (Vanderschuren and Kalivas, 2000; Vezina and Suto, 2003) and that CaMKII can regulate AMPA receptor signaling in NAcc neurons (Anderson et al., 2008; Sun et al., 2008).

Exposure to cocaine transiently increases αCaMKII levels in NAcc (Boudreau et al., 2009). Because sensitization is long-lasting, such transient increases cannot account for long-lasting enhancements in responding even though expression of sensitization continues to require CaMKII activation. However, given the role played by αCaMKII in synaptic plasticity (Lisman et al., 2002), it is likely that even a transient increase in αCaMKII can initiate long-lasting neuroadaptations and that some of these are responsible for the maintenance of sensitization. Here, we demonstrate that exposure to amphetamine also transiently increases αCaMKII levels in the NAcc and use viral-mediated gene transfer to show that transient overexpression of this protein in NAcc neurons produces long-lasting enhancements in behavioral responding to amphetamine.

Materials and Methods

Subjects. Male Sprague Dawley (locomotion experiments) and Long–Evans rats (self-administration experiments) weighing 250–275 g on arrival were purchased from Harlan and housed individually in a reverse cycle room (12 h light/dark) with food and water available *ad libitum*. All rats were afforded 4–5 d acclimation before the start of any experimental procedures. In some experiments, rats were anesthetized with a mix of ketamine (100 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.), placed in a stereotaxic instrument with the incisor bar positioned 5.0 mm above the interaural line (Pellegrino et al., 1979), and chronically implanted with bilateral guide cannulae (22 gauge; Plastics One) aimed either at the NAcc shell [anteroposterior (A/P), +3.4; lateral (L), ±0.8; dorsoventral (DV), −7.5], NAcc core (A/P, +3.4; L, ±1.5; DV, −7.5), or the ventral tegmental area (VTA) (A/P, −5.6; L, ±0.6; DV, −8.9). Coordinates are in millimeters from bregma and skull. Guide cannulae were angled at 10° (NAcc) or 16° (VTA) to the vertical and positioned 4 mm above the final injection site. After surgery, 28 gauge obturators were placed into the guide cannulae flush to the guide tips and rats were returned to their home cage for a 7–10 d recovery period. In other experiments, rats were also implanted with intravenous catheters 7–10 d after cannula implantation into the NAcc shell. These were made of SILASTIC tubing (Dow Corning), inserted into the right internal jugular vein, and positioned to exit slightly caudal to the midscapular region. Self-administration testing began 5–7 d later. Catheters were flushed daily with a sterile 0.9% saline solution containing 30 IU/ml heparin and 250 mg/ml ampicillin to promote patency (Pierre and Vezina, 1997). All surgical procedures were conducted using aseptic techniques according to an approved Institutional Animal Care and Use Committee protocol.

Design and procedure. Rats in different groups were infused intracranially with herpes simplex virus (HSV) vectors to transiently overexpress αCaMKII or exposed to a sensitizing regimen of amphetamine injections. Starting 4 d later, HSV-infected rats were tested for their locomotor response to systemic amphetamine or NAcc AMPA. Other rats were tested for their self-administration of amphetamine before and after HSV infection. Rats in additional groups were killed soon (4 or 8 d) or long (2–3 weeks) after HSV infection or exposure to amphetamine, and brain sections harvested for assessment of protein levels using Western immunoblotting or visualization of infection patterns using immunohistochemistry.

Exposure to amphetamine. Rats were administered five injections of amphetamine (1.5 mg/kg, i.p.) or saline (1.0 ml/kg, i.p.), one injection every third day. In all cases, rats were transported in their housing cages to a distinctive experimental room, administered their respective injections, placed back in their housing cages, and returned to the colony room 2 h later. This regimen of amphetamine injections is well established to sensitize drug-induced locomotion, NAcc DA overflow, and drug self-administration (Vezina, 2004).

Viral-mediated gene transfer. Replication-deficient viral vectors were constructed and packaged as described by Neve et al. (1997). Briefly, cDNA was inserted into the HSV amplicon HSV-PrpUC, packaged with the replication-deficient IE2 deletion mutant 5*dl*1.2 helper virus derived from the KOS strain, and resuspended in 10% sucrose. The average titer of the resulting viral stocks was 4.0×10^7 infectious units/ml. Transgene expression was regulated by HSV IE 4/5 (see Fig. 2*A*). These HSV vectors were used because they produce a transient increase in transgene expression that is maximal at 3–4 d and dissipates 7–8 d after infection (Carlezon et al., 1997; Neve et al., 1997).

Of the four different identified CaMKII subunits, the α and β subunits are the principal isoforms found in brain, in which they form dodecameric holoenzymes (Bennett et al., 1983; Miller and Kennedy, 1986). Because α is the predominant isoform in rat forebrain (the α/β ratio is ~3:1) (Bennett et al., 1983; Kennedy et al., 1983), the αCaMKII subunit was studied in the present experiments. The following constructs were used: αCaMKII (wild type), T286D αCaMKII (a constitutively active form created by replacing the threonine residue with aspartate), αCaMKII-GFP (either fusion or separate DNA), green fluorescent protein (GFP), and LacZ. The separate DNA αCaMKII-GFP construct and αCaMKII were used interchangeably in the behavioral and protein experiments as they produced similar effects. Likewise, LacZ and GFP were used interchangeably as controls with the 10% sucrose vehicle in the behavioral and protein experiments as they were found to be without detectable effects.

After recovery from surgery, rats were transferred to a biosafety level 2 facility in which they were administered bilateral intracranial microinjections of sucrose vehicle or their respective viral vectors. Microinjections were made in freely moving rats in a volume of 2.0 μl/side at a rate of 0.1 μl/30 s through 28 gauge cannulae extending 4 mm beyond the guide cannulae tips. Microinjectors were connected via polyethylene tubing (PE20) to Hamilton syringes and left in place for 5 min after the injection to allow for diffusion. Rats were returned to the colony room 24 h later. All procedures were conducted according to an approved Institutional Biosafety Committee protocol.

Transient αCaMKII overexpression and locomotor responding to amphetamine. Four experiments were conducted to assess the effect of αCaMKII overexpression in different brain regions on locomotor responding to amphetamine. In all cases, rats were randomly assigned to one of two groups (infection with an αCaMKII construct or control) and tested for their locomotor response to a threshold dose of amphetamine (0.5 mg/kg, i.p.) 4, 8, 12, 16, 20, 27, and 34 d after infection. This permitted the establishment of a detailed time course of locomotor responding relative to the transient viral overexpression pattern.

In one experiment, αCaMKII was overexpressed bilaterally in the NAcc shell. In another, T286D αCaMKII, a constitutively active form of αCaMKII, was overexpressed in the NAcc shell. This experiment tested whether the αCaMKII overexpressed must be in the autophosphorylated state to produce an effect. To confirm the subregion specificity of the effects to the NAcc shell, an additional experiment tested the effect of αCaMKII overexpression in the NAcc core as well as sites adjacent to the NAcc. A fourth experiment tested the effect of αCaMKII overexpression in the VTA to directly assess the impact of DA neuron infection.

Locomotor activity was measured by using a bank of 12 activity boxes. Each box (22 × 43 × 33 cm) was constructed of opaque plastic (rear and two side walls), a Plexiglas front-hinged door, and a tubular stainless-steel ceiling and floor. Two photocells, positioned 2.5 cm above the floor and spaced evenly along the longitudinal axis of each box, were used to quantify locomotion. Separate interruptions of photocell beams were detected and recorded via an electrical interface by a computer situated in an adjacent room using locally developed software. On each test day, locomotor activity was measured 1 h before and 2 h after the amphetamine challenge injection.

Transient αCaMKII overexpression and locomotor responding to NAcc shell AMPA. Rats were randomly assigned to one of two groups (infection with an αCaMKII construct or control) and tested for their locomotor response to NAcc shell AMPA (0.4 nmol/0.5 μl per side) either soon (4 d)

or long (2–3 weeks) after infection. Microinjections were made in freely moving rats at a rate of 0.5 μl/30 s using the same guide cannulae used to deliver the viral vectors to the NAcc shell. Microinjectors were left in place for 1 min after the injection to allow for diffusion. Locomotor activity was measured 1 h before and 2 h after the challenge injection.

Transient αCaMKII overexpression in NAcc shell and amphetamine self-administration. After recovery from surgery (3–5 d for intravenous catheters; ~2 weeks for intracranial cannulae), rats were trained to self-administer amphetamine on fixed-ratio (FR) schedules of reinforcement and then tested under a progressive ratio (PR) schedule for 4 d. The following day, rats were transferred to a biosafety level 2 facility in which they were randomly assigned to one of two groups (NAcc shell infection with αCaMKII or control). On return of the rats to the colony room the following day, PR self-administration testing resumed and continued for up to 2 weeks after infection.

Sixteen test chambers (Coulbourn Instruments) (31 × 25 × 30 cm) were used. Each was equipped with a retractable lever (6 cm above the floor), a stimulus light (13 cm above the lever), a counterbalanced arm, a steel-spring tether, and an infusion pump (model PHM-100; MED Associates) that allowed free movement of the animal in the chamber and drug delivery on depression of the lever. Lever presses and drug infusions were recorded and controlled via an electrical interface by a computer using MED Associates software. Amphetamine self-administration sessions were of a maximum 3 h duration and conducted daily as described by Vezina et al. (2002). Briefly, reinforced lever presses delivered an infusion of amphetamine (200 μg/kg/infusion) through the intravenous catheter. During training under the FR schedules, an experimenter-delivered amphetamine priming infusion was given at the beginning of each session, and rats were required to then self-administer 10 infusions of amphetamine in 3 h first on an FR1 and then on an FR2 schedule of reinforcement. Animals that did not satisfy each of the FR1 and FR2 criteria within 5 d were excluded from the study. A total of 15 rats were thus excluded. Days to satisfaction of the training criteria under each FR schedule were recorded. Successful rats were subsequently tested under a PR schedule in which the number of responses required to obtain each successive infusion of amphetamine was determined by ROUND [5 × EXP(0.25 × infusion number) − 5] to produce the following sequence of required lever presses: 1, 3, 6, 9, 12, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc. (Richardson and Roberts, 1996). The daily PR test sessions were terminated after 3 h or after 1 h elapsed without a drug infusion. Priming amphetamine infusions were not given on these sessions. The number of infusions obtained in each PR session was recorded. Catheter viability was assessed throughout testing. A total of 15 rats were excluded because of catheters that lost patency or developed leaks.

Amphetamine exposure and NAcc signaling. To determine the effect of amphetamine exposure on αCaMKII and pαCaMKII protein levels as well as AMPA receptor signaling in the NAcc, rats were killed soon (4 d) or long (2–3 weeks) after the last exposure injection of amphetamine or saline, and their brains were rapidly removed for immunoblotting. αCaMKII, pαCaMKII (Thr286), GluR1, pGluR1 (Ser831), pGluR1 (Ser845), and GluR2 levels were assessed in both the NAcc core and shell.

Transient αCaMKII overexpression and signaling in NAcc shell. To determine the effect of transient αCaMKII overexpression on signaling in NAcc shell, rats were killed soon (4 or 8 d) or long (2–3 weeks) after NAcc shell infection, and their brains were rapidly removed for immunoblotting. αCaMKII and pαCaMKII (Thr286) levels were determined to verify the amount and time course of viral-mediated overexpression. GluR1, pGluR1 (Ser831), pGluR1 (Ser845), and GluR2 levels were assessed to identify αCaMKII-mediated alterations in AMPA receptor signaling both when αCaMKII protein levels were elevated and long after they had returned to baseline. As αCaMKII influences CREB activity, CREB activity indirectly influences cdk5 levels, and both CREB and cdk5 signaling regulate behavioral responding to drugs (Bibb et al., 2001; Benavides and Bibb, 2004; Carlezon et al., 2005), pCREB (Ser142; CaMKII site), pCREB (Ser133; stimulatory site), total CREB, and cdk5 levels were also assessed.

Immunoblotting. Brains were removed rapidly and flash-frozen on dry ice. For amphetamine-exposed rats, sections (1 mm thick) were obtained with a brain matrix and a skewed donut punch approach used to produce punches of the NAcc core (1-mm-diameter punch) and the medial and ventral NAcc shell (2 mm crescent punch). For rats infected in the NAcc shell, sections (1 mm thick) were obtained with a brain matrix, and 2-mm-diameter punches were taken bilaterally around the injection cannula tips. Tissue punches were frozen on dry ice and processed as described by Carlezon and Neve (2003) and Jiao et al. (2007). Briefly, tissue was homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails (1 and 2; Sigma-Aldrich), and protein levels were measured by the Bradford method. A total of 20 μg of protein was loaded per lane and separated by 10% SDS-PAGE. After transfer, membranes were incubated in blocking solution (5% milk in Tris-buffered saline containing 0.1% Tween) (TBS-T) sequentially containing no antibody, primary antibodies for αCaMKII (1:1000; Millipore), pαCaMKII (Thr286; 1:1000; Millipore), GluR1 (1:1000; Millipore), pGluR1 (Ser831; 1:500; Millipore), pGluR1 (Ser845; 1:500; Millipore), GluR2 (1:1000; Millipore), CREB (1:1000; Cell Signaling Technologies), pCREB (Ser142; 1:500; generously provided by Dr. Michael Greenberg, Harvard University, Boston, MA) (Kornhauser et al., 2002), pCREB (Ser133; 1:1000; Millipore), or cdk5 (1:1000; Santa Cruz), and a HRP-conjugated anti-mouse or rabbit IgG (Jackson ImmunoResearch Laboratories). Bands were visualized using the ECL detection system (ECL Advanced; GE Healthcare). Membranes were then stripped and probed with a mouse-derived antibody for β-actin as a loading control (1:2000; Sigma-Aldrich), incubated in a HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories), and developed as above.

Immunofluorescence. Separate rats were used in immunohistochemistry studies to visualize the pattern of infection and determine the extent of transport of the virus, if any. Four days after infection in the NAcc shell with the fusion αCaMKII-GFP construct to allow direct assessment of αCaMKII localization, brains were harvested, flash-frozen in chilled isopentane, and stored at −80°C. To detect GFP fluorescence, 20 μm sections were prepared, mounted in ProGold antifade mountant (Invitrogen), and analyzed using a confocal fluorescence microscope with an argon–krypton laser and appropriate performance filters. The distribution pattern of GFP-positive cells around the injection cannula tips was assessed. Entire brains (from midbrain to prefrontal cortex) were also examined for GFP-positive cells to determine the extent of anterograde and retrograde transport of the virus from the NAcc shell microinjection site. The nonfluorescent control vector HSV-LacZ that encodes β-galactosidase was also used to visualize infection patterns around injection cannula tips. Brains were harvested, and tissue was processed as described by Carlezon and Neve (2003). To detect β-galactosidase, a 0.2 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside solution was used (Thermo Fisher Scientific).

Drugs. *S*(+)-Amphetamine sulfate and AMPA were obtained from Sigma-Aldrich. Drugs were dissolved in sterile saline and dH_2O for intraperitoneal and intracranial routes of administration, respectively. Doses refer to the weight of the salt.

Histology. After the completion of the behavioral experiments, rats were deeply anesthetized and perfused transcardially with 0.9% saline and 10% formalin. Brains were removed and stored in the formalin solution for at least 24 h. The 40 μm sections were then prepared, mounted on gelatin-coated slides, and stained with cresyl violet to identify rats with injection cannula tips located bilaterally in the NAcc shell, NAcc core, sites medial, lateral or rostral to the NAcc, or in the VTA. Only rats with both cannula tips in the targeted region were retained for statistical analyses and their number subsequently indicated as *n*/group. The number of rats that failed to meet this criterion after NAcc shell infection was as follows: amphetamine-induced locomotion: αCaMKII, 0; control, 2; T286D αCaMKII, 2; control, 3; NAcc AMPA-induced locomotion: αCaMKII, 7; control, 2; amphetamine self-administration: αCaMKII, 3; control, 2. Four αCaMKII and six control rats failed to meet this criterion after VTA infection.

Data analyses. Locomotor test data were analyzed with between-within ANOVA with group (αCaMKII or control) as the between factor and days (7) or time (6 and 12) as the within factor. *Post hoc* comparisons after ANOVA were made using the Scheffé test. In the self-administration experiment, five rats experienced failed catheters at different points over the course of PR self-administration testing, precluding analysis by repeated-measures ANOVA of the entire 14 d after infection. Thus,

independent-samples *t* tests were conducted on the number of infusions obtained by αCaMKII and control rats averaged over the 4 d before infection and the days completed after infection. The number of infusions obtained, rather than the number of presses emitted or final ratio achieved, was analyzed because the latter were derived by definition from an exponential function (Richardson and Roberts, 1996). For the immunoblot data, all bands were normalized to β-actin and protein/β-actin ratios analyzed with independent-samples *t* tests (amphetamine-exposed, αCaMKII, or controls). These data were expressed graphically as the average percentage change from control group means (set at 100%).

Results

Exposure to amphetamine transiently increases αCaMKII in NAcc shell

Consistent with a role for the NAcc shell in the expression of sensitization (Pierce and Kalivas, 1995; Loweth et al., 2008), exposure to a sensitizing regimen of amphetamine injections led to a transient increase in αCaMKII protein levels in the shell but not the core of the NAcc (Fig. 1*A*,*B*). αCaMKII levels were significantly increased in amphetamine- relative to saline-exposed rats in the NAcc shell (shell: $t_{(10)} = 3.11$, $p < 0.01$; core: $t_{(9)} = 0.175$, ns) 4 d but not 2–3 weeks after exposure (shell: $t_{(12)} = 0.289$, ns; core: $t_{(8)} = 0.502$, ns). No changes were observed in pαCaMKII levels in either region at either withdrawal time (data not shown). Thus, when examined under basal conditions, the transient increase in total αCaMKII protein observed was not accompanied by a detectable change in pαCaMKII (Thr286) levels, a site that, when phosphorylated, allows the kinase to remain active in the absence of Ca^{2+}/calmodulin (Lisman et al., 2002).

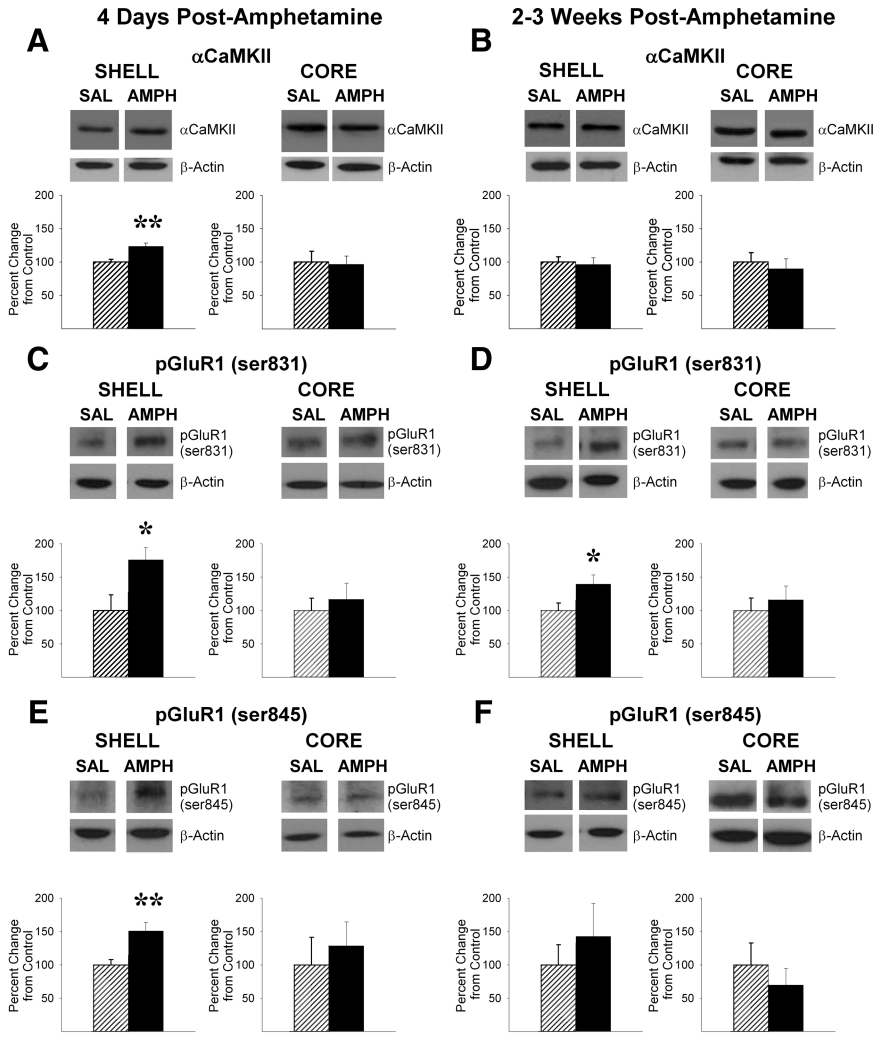

Figure 1. Exposure to amphetamine increases αCaMKII and pGluR1 in NAcc shell. Rats were exposed to amphetamine (AMPH) or saline (SAL) and killed 4 d or 2–3 weeks later. Protein levels obtained in the NAcc shell or core are shown as group mean (+SEM) percentage change from controls with representative immunoblots. In the NAcc shell, amphetamine exposure produced a transient increase in αCaMKII (***A***, ***B***) and pGluR1 (Ser845) (***E***, ***F***). Long-lasting increases in pGluR1 (Ser831) were observed in this site (***C***, ***D***). No significant effects were detected in the NAcc core. *$p < 0.05$, **$p < 0.01$, AMPH-exposed (black bars) versus SAL-exposed (cross-hatched bars). $n = 4–8$/group.

Exposure to amphetamine increases pGluR1 in NAcc shell

Because αCaMKII regulates AMPA receptor signaling and the latter contributes to the expression of sensitization (Vanderschuren and Kalivas, 2000; Vezina and Suto, 2003), the NAcc was examined for alterations in AMPA receptor protein levels soon and long after exposure to amphetamine. Both the GluR1 αCaMKII (Ser831) and protein kinase A (PKA) (Ser845) sites were examined as increased phosphorylation at either site enhances AMPA receptor transmission (Song and Huganir, 2002). Under the present conditions, no changes were detected in either region at either withdrawal time in total protein levels of either GluR1 or GluR2 AMPA receptor subunit. However, consistent with the increase in αCaMKII 4 d after exposure to amphetamine, pGluR1 (Ser831) was significantly increased in the NAcc shell at this time relative to saline-exposed controls ($t_{(6)} = 2.51$; $p < 0.05$) (Fig. 1*C*). Significant increases in pGluR1 (Ser845) levels were also observed at this time in the NAcc shell ($t_{(7)} = 3.15$; $p < 0.01$) (Fig. 1*E*). Both effects are consistent with a role for AMPA receptor-mediated glutamatergic transmission in the expression of sensitization by cocaine at early withdrawal times (Pierce et al., 1996b). Levels of pGluR1 (Ser831), but not pGluR1 (Ser845), remained significantly elevated in the NAcc shell 2–3 weeks after amphetamine exposure ($t_{(7)} = 2.28$; $p < 0.05$) (Fig. 1*D*,*F*), again consistent with a continued role for glutamatergic transmission in the expression of sensitization at long withdrawal periods (Kim et al., 2005). No significant group differences were detected at either time point in the NAcc core.

Viral-mediated gene transfer leads to transient localized overexpression of the transgene

Consistent with previous findings obtained with the HSV vector system (Carlezon et al., 1997; Neve et al., 1997), microinjections of HSV-αCaMKII into the NAcc shell led to a transient and localized increase in transgene expression. Protein immunoblot analyses revealed a significant increase in αCaMKII protein levels in the NAcc shell at day 4 ($t_{(4)} = 2.66$; $p < 0.05$) but no longer at day 8 ($t_{(4)} = 0.05$; ns) or 2–3 weeks ($t_{(9)} = 1.47$; ns) after infection (Fig. 2*A*). A small but significant increase in pαCaMKII was observed at day 4 after infection ($t_{(8)} = 2.05$; $p < 0.05$), a potential consequence of the substantial αCaMKII overexpression at this

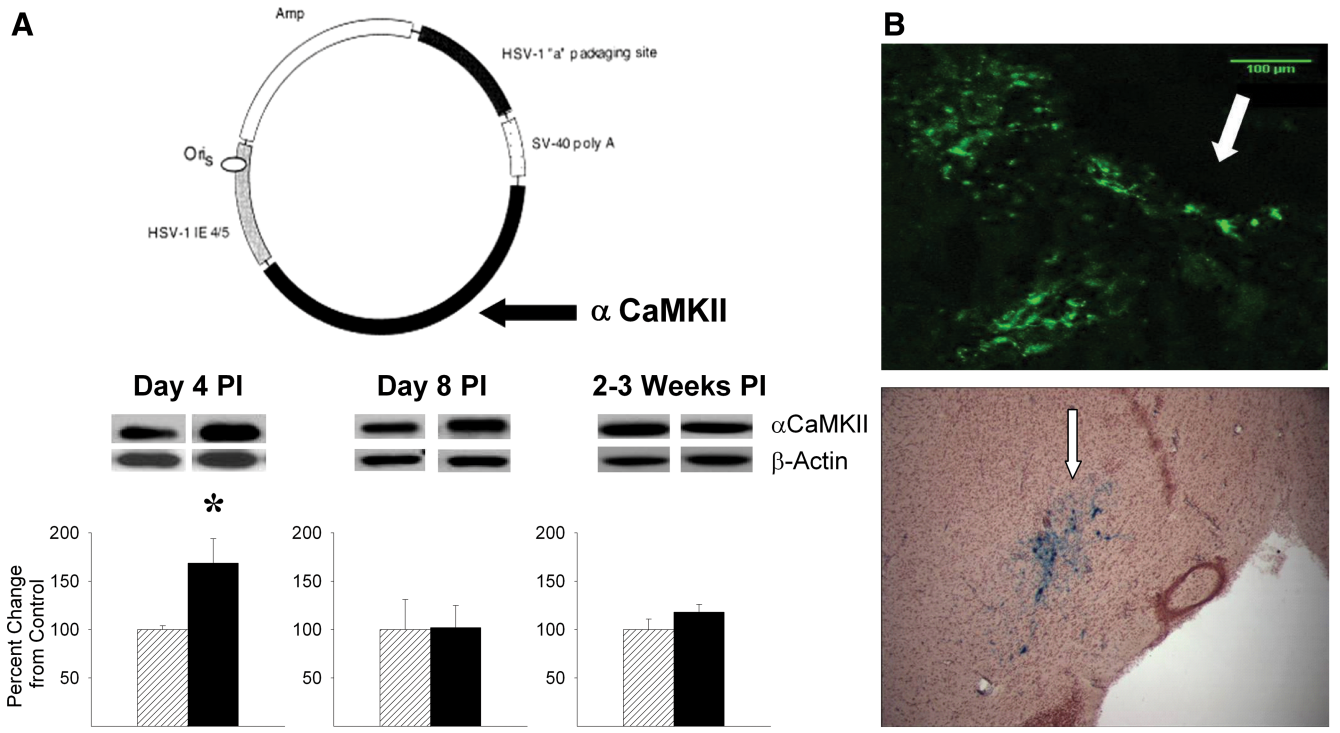

Figure 2. Viral-mediated transient overexpression of αCaMKII in the NAcc shell. ***A***, Western blots of αCaMKII obtained 4 d, 8 d, and 2–3 weeks postinfection (PI). αCaMKII was maximally and only elevated 4 d PI. Protein levels are expressed as group mean (+SEM) percentage change from controls ($n = 3–6$/group). *$p < 0.05$, αCaMKII (black bars) versus control (cross-hatched bars). The schema illustrates the components of the HSV amplicon used to overexpress αCaMKII. ***B***, Photomicrographs of the NAcc obtained 4 d after infection with HSV-αCaMKII-GFP (top) or HSV-LacZ (bottom) illustrating GFP- or β-galactosidase-positive neurons in close proximity to the injection cannula tips (arrows).

time. No significant increase was observed 2–3 weeks after infection (data not shown).

Immunohistological examination of brain sections 4 d after infection with the fusion αCaMKII-GFP construct revealed transgene expressing cells in close proximity to the site of injection in the NAcc, a pattern also observed with the localized overexpression of β-galactosidase after microinjection of the control virus HSV-LacZ (Fig. 2*B*). CaMKII-GFP-expressing cells exhibited large dendritic processes consistent with the morphology of medium spiny neurons in the NAcc and the neuron-preferring characteristics of HSV (Carlezon et al., 2000). Whole-brain immunohistochemical analyses performed in seven rats revealed that infection was limited to these neurons in the NAcc and that neurons in nuclei projecting to this site were mostly spared. Only one GFP-positive cell outside the NAcc was located in the VTA, indicating little to no retrograde transport of the virus. As previously observed with this HSV vector (Neve et al., 1997), no significant toxicity was observed.

Transient αCaMKII overexpression in the NAcc shell enhances amphetamine-induced locomotion

The transient protein overexpression afforded by the HSV vector system was used to mimic in intrinsic NAcc cells the transient increase in αCaMKII observed in the NAcc shell after exposure to amphetamine. On day 4 after infection, when αCaMKII protein levels were elevated, HSV-αCaMKII-infected rats showed an enhanced locomotor response to amphetamine compared with control rats. Remarkably, HSV-αCaMKII-infected rats continued to show enhanced responding up to 34 d after infection, long after αCaMKII protein levels had returned to baseline (Fig. 3). The ANOVA conducted on the group mean 2 h total locomotor counts obtained after amphetamine on the 7 test days revealed an overall group effect ($F_{(1,15)} = 25.78$; $p < 0.001$). Significant group differences were confirmed on each test day by *post hoc* Scheffé comparisons ($p < 0.05–0.001$). No significant effect of days or group by days interaction were detected. As shown in the control rats, the threshold dose of amphetamine used (0.5 mg/kg, i.p.) does not lead to sensitization with repeated injection. These findings indicate that αCaMKII acts in intrinsic NAcc shell neurons to enhance amphetamine-induced locomotor activity in at least two ways: directly when αCaMKII levels are elevated and via postphosphorylation cascades that lead to long-term neuroadaptations in this site. Transient overexpression of αCaMKII at no time increased locomotor responding significantly in the absence of amphetamine. The ANOVA conducted on the group mean 1 h total locomotor counts obtained before the amphetamine challenge injection on the 7 test days revealed no significant group effect ($F_{(1,15)} = 0.94$; ns) and no significant group by days interaction ($F_{(6,90)} = 1.18$; ns).

Similar findings were obtained when the constitutively active construct T286D αCaMKII was overexpressed in the NAcc shell of separate rats (Table 1), indicating that the autophosphorylation state of the overexpressed αCaMKII is not a determining factor for enhancing amphetamine-induced locomotion. The effects of αCaMKII overexpression were also specific to the NAcc shell and attributable to infection of intrinsic neurons in this site as overexpressing αCaMKII in the NAcc core, regions adjacent to the NAcc (Table 2), or in the VTA (Table 3) did not increase amphetamine-induced locomotion.

Transient αCaMKII overexpression enhances locomotor responding to NAcc shell AMPA

To begin investigating the neuroadaptations underlying the enhanced amphetamine-induced locomotion observed both

soon and long after infection with HSV-αCaMKII, locomotor responding to NAcc shell AMPA (0.4 nmol/0.5 μl per side) was assessed 4 d and 2–3 weeks after transiently overexpressing αCaMKII in this site. Compared with controls, NAcc shell AMPA increased locomotion to a significantly greater extent both 4 d and 2–3 weeks after infection (Fig. 4). The ANOVA conducted on these data revealed an overall effect of group ($F_{(1,14)} = 4.82$; $p < 0.05$) at day 4 and an overall group effect ($F_{(1,18)} = 7.88$; $p < 0.05$), a significant effect of time ($F_{(11,198)} = 9.94$; $p < 0.001$), and a significant group by time interaction ($F_{(11,198)} = 2.58$; $p < 0.01$) at 2–3 weeks. These results indicate that transiently overexpressing αCaMKII in the NAcc leads to functional upregulation of AMPA receptors both when protein levels are increased and long after protein levels have returned to baseline. Again, transient overexpression of αCaMKII did not significantly increase locomotor responding in the period before the AMPA challenge either 4 d or 2–3 weeks after infection. The ANOVA conducted on these data revealed only significant effects of time.

Transient αCaMKII overexpression in the NAcc shell enhances amphetamine self-administration

Rats trained to self-administer amphetamine were tested under a PR schedule for 4 d, administered HSV-αCaMKII or control infusions into the NAcc shell, and tested under the PR schedule for an additional 14 d (Fig. 5). No significant differences between rats randomly assigned to the αCaMKII or control conditions were observed on the first 4 preinfection test days ($t_{(14)} = 0.12$; ns). However, by day 4 after infection, when αCaMKII protein levels were significantly elevated, HSV-αCaMKII-infected rats began working more and thus obtained significantly more amphetamine infusions compared with controls ($t_{(14)} = 2.57$; $p < 0.05$). As observed with amphetamine-induced locomotion, enhanced self-administration was maintained for an additional 10 d of testing, although αCaMKII rats exhibited some variability during this period. Analysis of the number of infusions obtained averaged over the days completed from day 4 after infection showed that αCaMKII rats nonetheless self-administered significantly more amphetamine relative to control rats ($t_{(14)} = 2.03$; $p < 0.05$). These results indicate that transient overexpression of αCaMKII in the NAcc shell leads to a long-lasting enhancement in amphetamine self-administration that begins 4 d after infection, when protein levels are maximally elevated, and is maintained long after αCaMKII levels have returned to baseline.

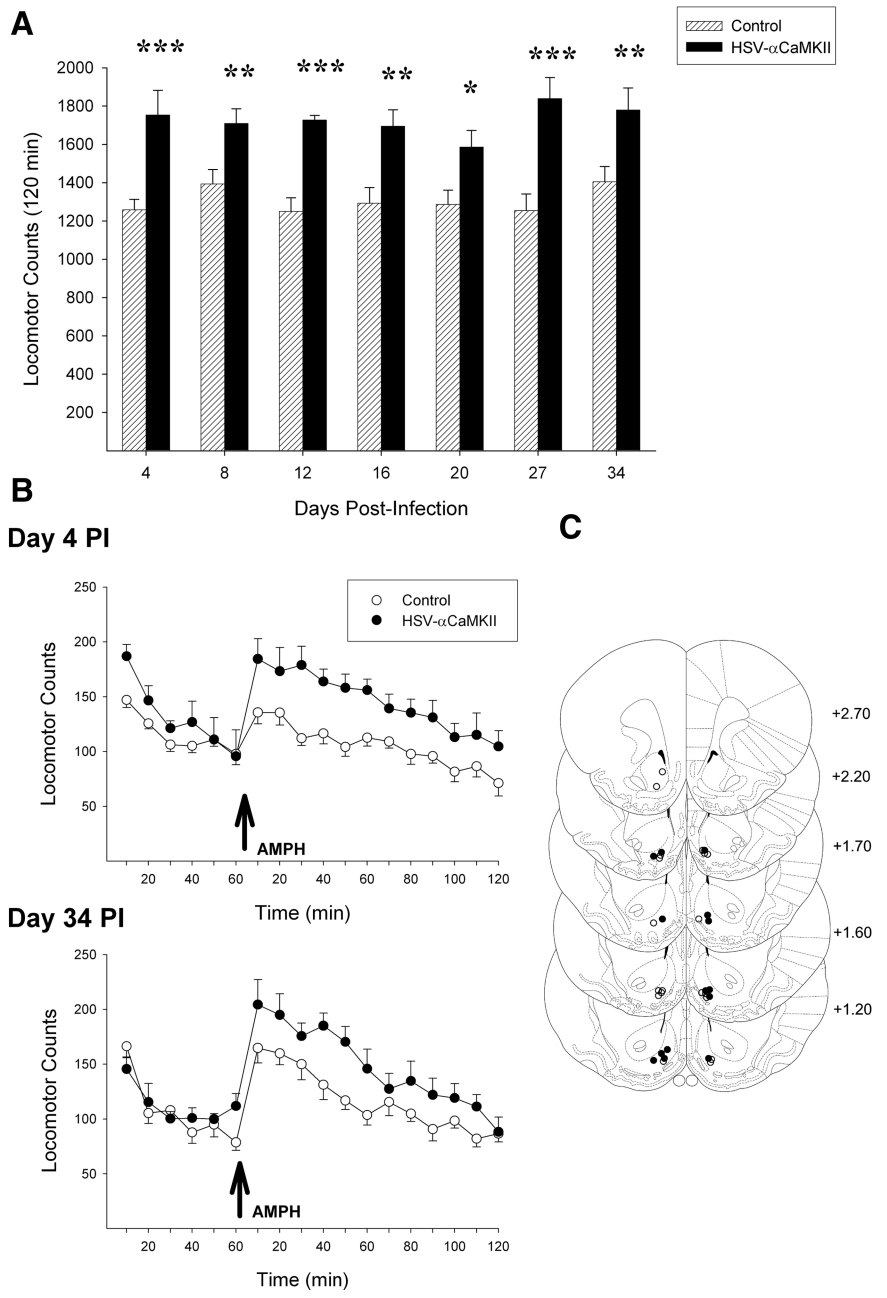

Figure 3. Transient αCaMKII overexpression in the NAcc shell enhances locomotor responding to amphetamine. ***A***, Data are shown as group mean (+SEM) 2 h total locomotor counts observed after the amphetamine injections on the different test days postinfection (*n*/group = 7–10). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, significantly different from controls at specified day. ***B***, Time course of the enhanced locomotor response to amphetamine observed 4 and 34 d after infection. Data are shown as group mean locomotor counts (±SEM) before and after the amphetamine injection (arrows at abscissas). ***C***, Location of the injection cannula tips for rats included in the data analyses. The symbols refer to group affiliation (○, controls; ●, αCaMKII). The line drawings are from the work by Paxinos and Watson (1997). The numbers to the right indicate millimeters from bregma.

Transient αCaMKII overexpression increases pGluR1 in NAcc shell

Compared with controls, αCaMKII overexpression in the NAcc shell significantly increased pGluR1 (Ser831) levels 4 d after infection ($t_{(8)} = 2.65$; $p < 0.05$), when αCaMKII protein levels were elevated, as well as 2–3 weeks after infection ($t_{(14)} = 2.61$; $p < 0.05$), long after αCaMKII protein levels had returned to baseline (Fig. 6*A*). Thus, as with amphetamine exposure, transient αCaMKII overexpression in the NAcc shell produced a long-lasting increase in pGluR1 (Ser831) in this site that could enhance

Table 1. Transient overexpression of T286D αCaMKII in the NAcc shell enhances amphetamine-induced locomotion

Group	Day 4 PI	Day 8 PI	Day 12 PI	Day 16 PI	Day 20 PI	Day 27 PI	Day 34 PI
NAcc shell T286D ($n = 8$)	1640 * (±97)	1760 ** (±116)	1673 ** (±137)	1656 (±86)	1605 * (±97)	1608 * (±155)	1505 * (±101)
NAcc shell control ($n = 8$)	1321 (±96)	1343 (±99)	1186 (±116)	1371 (±78)	1277 (±120)	1237 (±122)	1100 (±101)

Data are shown as group mean (±SEM) 2 h total locomotor counts observed after amphetamine (0.5 mg/kg, i.p.) on the different test days postinfection (PI) in HSV-T286D αCaMKII-infected rats and controls. The ANOVA revealed an overall group effect ($F_{(1,14)} = 11.57$; $p < 0.005$).

*$p < 0.05$, **$p < 0.01$, significant group difference revealed by *post hoc* Scheffé comparisons.

Table 2. Transient overexpression of αCaMKII in the NAcc core or regions adjacent to the NAcc does not enhance amphetamine-induced locomotion

Group	Day 4 PI	Day 8 PI	Day 12 PI	Day 16 PI	Day 20 PI	Day 27 PI	Day 34 PI
NAcc core αCaMKII ($n = 6$)	1396 (±98)	1136 (±47)	1149 (±63)	1268 (±104)	1220 (±97)	1401 (±80)	1516 (±141)
NAcc core control ($n = 5$)	1319 (±181)	1130 (±132)	1048 (±77)	1356 (±154)	1281 (±124)	1359 (±148)	1380 (±121)
Adjacent NAcc αCaMKII ($n = 5$)	1077 (±72)	1284 (±77)	1105 (±124)	1117 (±47)	1180 (±113)	1136 (±89)	1121 (±94)

Data are shown as described in Table 1. The ANOVA revealed only a significant effect of time. Rats in the Adjacent NAcc αCaMKII group received infusions of HSV-αCaMKII in sites medial, lateral, or rostral to the NAcc. PI, Postinfection.

Table 3. Transient overexpression of T286D αCaMKII in the VTA does not enhance amphetamine-induced locomotion

Group	Day 4 PI	Day 8 PI	Day 12 PI	Day 16 PI	Day 20 PI	Day 27 PI	Day 34 PI
VTA T286D ($n = 7$)	1548 (±282)	1557 (±239)	1514 (±163)	1419 (±186)	1557 (±208)	1506 (±246)	1396 (±259)
VTA control ($n = 5$)	1549 (±314)	1756 (±256)	1698 (±145)	1531 (±227)	1653 (±162)	1599 (±177)	1531 (±189)

Data are shown as described in Table 1. The ANOVA revealed no significant effects. Similar findings were obtained with a smaller group of rats infected with HSV-αCaMKII. PI, Postinfection.

behavioral output by increasing AMPA receptor conductance (Song and Huganir, 2002). No significant changes were observed in phosphorylation at the GluR1 (Ser845) PKA site or in total GluR1 or GluR2 levels either soon or long after infection.

Transient αCaMKII overexpression increases pCREB (Ser142) in NAcc shell

Levels of pCREB (Ser142; an αCaMKII inhibition site) (Wu and McMurray, 2001) were increased 4 d after infection when αCaMKII levels were elevated ($t_{(11)} = 3.26$; $p < 0.01$) but not 2–3 weeks later ($t_{(10)} = 1.55$; ns). No significant changes were observed at either time point in pCREB (Ser133) or in total CREB and cdk5 levels (data not shown). Thus, the transient increase in αCaMKII appears to have led to a corresponding transient inhibition of CREB that could have enhanced behavioral responding to amphetamine soon after infection as suggested by previous reports (Carlezon et al., 1998; Pliakas et al., 2001). However, no long-lasting changes in these signaling pathways were detected after transient αCaMKII overexpression.

Discussion

Exposing rats to a sensitizing regimen of amphetamine injections transiently increased αCaMKII levels in the NAcc shell. Using a transient protein overexpression HSV vector system, we show that this increase in αCaMKII produces long-lasting enhancements in amphetamine-induced locomotion and self-administration manifested when αCaMKII levels are elevated and persisting long after they have returned to baseline. These findings demonstrate that αCaMKII in the NAcc shell can enhance behavioral responding to amphetamine in at least two different ways: directly when kinase levels are elevated and via postphosphorylation cascades that lead to long-lasting neuroadaptations in the NAcc. These findings provide insight into the different ways NAcc αCaMKII can contribute to the expression of psychostimulant sensitization. Research on the role of CaMKII has focused on its regulation of DA release from presynaptic DA terminals in the NAcc and striatum (Iwata et al., 1997; Pierce and Kalivas, 1997; Pierce et al., 1998; Kantor et al., 1999). The present experiments used HSV vectors to focus on the role played by αCaMKII in intrinsic NAcc shell neurons. Notably, transgene-expressing cells exhibiting the morphology of medium spiny neurons were restricted to the site of injection in the NAcc shell. Rats infected in the NAcc core, forebrain sites outside the NAcc, or the VTA did not show enhanced locomotor responding to amphetamine. These findings indicate that the enhanced amphetamine-induced locomotion and self-administration observed in infected rats was produced by transiently overexpressing αCaMKII in intrinsic NAcc shell neurons. The resulting long-lasting neuroadaptations in these neurons could contribute to the long-lasting maintenance of psychostimulant sensitization. The localization of the observed effects to the NAcc shell is consistent with the processing by neurons in this site of the psychomotor activating and incentive motivational properties of psychostimulant drugs (Everitt and Robbins, 2005). Indeed, the enhanced work output aimed at self-administering amphetamine observed in HSV-αCaMKII-infected rats supports a critical role for these neurons in mediating incentive sensitization, whether it is expressed as enhanced locomotion or enhanced drug intake (Robinson and Berridge, 1993).

Increased pαCaMKII levels were not observed after amphetamine exposure, and similar effects were produced by αCaMKII and T286D αCaMKII overexpression, suggesting that the autophosphorylation state of αCaMKII is not a determining factor for enhanced behavioral responding to amphetamine. Alternatively, αCaMKII phosphorylates a wide array of downstream targets and could modulate behavioral responding to amphetamine by altering a number of postreceptor pathways in NAcc neurons. For example, CaMKII can modulate D_3 DA receptors to diminish their ability to inhibit cocaine-induced locomotion (Liu et al., 2009). CaMKII also can inhibit CREB activity (Wu and McMurray, 2001), an effect known to increase cocaine reward (Carlezon et al., 1998; Pliakas et al., 2001). Overexpressing CaMKII could thus increase behavioral responding to amphetamine soon after infection by inhibiting CREB activity, a possibility supported by the increased pCREB (Ser142) levels observed in αCaMKII-infected rats in the present experiments. This effect required increased αCaMKII levels as it was not observed long after infection. Indeed, no change in pCREB (Ser133), total CREB, or cdk5 levels was observed either soon or long after infection, making it un-

likely that long-term changes in CREB production or phosphorylation could account for the long-lasting increases in behavioral responding to amphetamine observed.

CaMKII also regulates AMPA receptor function in a number of ways (Lisman et al., 2002), and AMPA receptor-mediated glutamate transmission plays an important role in the expression of sensitization (Wolf, 1998; Vanderschuren and Kalivas, 2000; Vezina and Suto, 2003). AMPA receptor antagonists block the expression of sensitization by amphetamine (Karler et al., 1991; Tzschentke and Schmidt, 1997; Mead and Stephens, 1998; cf. Li et al., 1997) and cocaine (Pierce et al., 1996a; Jackson et al., 1998; Bell et al., 2000; cf. Karler et al., 1994). In addition, NAcc AMPA produces enhanced locomotion and reinstatement of drug seeking in psychostimulant-sensitized rats (Pierce et al., 1996a; Suto et al., 2004). Interestingly, in the last two studies, AMPA was effective in the NAcc core and shell, suggesting with the present findings that different presynaptic and postsynaptic mechanisms may be recruited in these two subnuclei. Cocaine-sensitized rats also display increased surface expression of GluR1 and GluR2 AMPA receptor subunits in the NAcc (Boudreau and Wolf, 2005; Boudreau et al., 2007, 2009). Although a similar increase in AMPA receptor surface expression is not observed in amphetamine-sensitized rats (Nelson et al., 2009), other changes in AMPA receptor function can mediate the known contribution of glutamate transmission to the expression of sensitization by amphetamine (Karler et al., 1991; Tzschentke and Schmidt, 1997; Kim et al., 2005). In the present experiments, overexpressing αCaMKII in the NAcc shell enhanced locomotor responding to NAcc AMPA and increased pGluR1 (Ser831) levels at both early and late time points after infection. A similar increase in pGluR1 (Ser831) levels was observed soon and long after exposure to amphetamine. Phosphorylation of GluR1 (Ser831) increases channel conductance in GluR2-lacking AMPA receptors (Oh and Derkach, 2005). These receptors are expressed at relatively low levels in the NAcc (Boudreau et al., 2007), although their contribution to synaptic transmission in drug-naive animals remains unclear. Experiments assessing AMPA receptor current–voltage relationships and the effect of selectively blocking these receptors have revealed a small contribution to synaptic transmission in the NAcc shell of adult (Campioni et al., 2009) but not young mice (Kourrich et al., 2007; Mameli et al., 2009) and none in the NAcc core of adult rats (Conrad et al., 2008). However, after exposure to cocaine, levels of GluR2-lacking AMPA receptors are increased in the NAcc (Conrad et al., 2008) as is their contribution to synaptic transmission in the NAcc shell (Mameli et al., 2009; cf. Kourrich et al., 2007) and core (Conrad et al., 2008). Thus, the enhanced locomotor responding to NAcc AMPA and amphetamine observed here may have resulted from increased conductance in GluR2-lacking AMPA receptors caused by increased phosphorylation of the Ser831 residue by αCaMKII. Together, these findings indicate that exposure to amphetamine and viral-mediated overexpression of αCaMKII produce similar neuroadaptations in the NAcc shell that may contribute to enhanced behavioral responding to the drug. A small but significant increase in GluR1 protein was reported selectively in the NAcc shell after exposure to high concentrations of amphetamine in the rat (Nelson et al., 2009). As this increase was not accompanied by a change in GluR2 protein, such a regimen may also lead to an increase in GluR2-lacking AMPA receptors in this site. Other evidence indicates that altering specific AMPA receptor subunit levels in the NAcc may differentially affect behavioral responding to psychostimulants: elevated GluR1 levels are associated with aversion, and, conversely, elevated GluR2 levels with enhanced

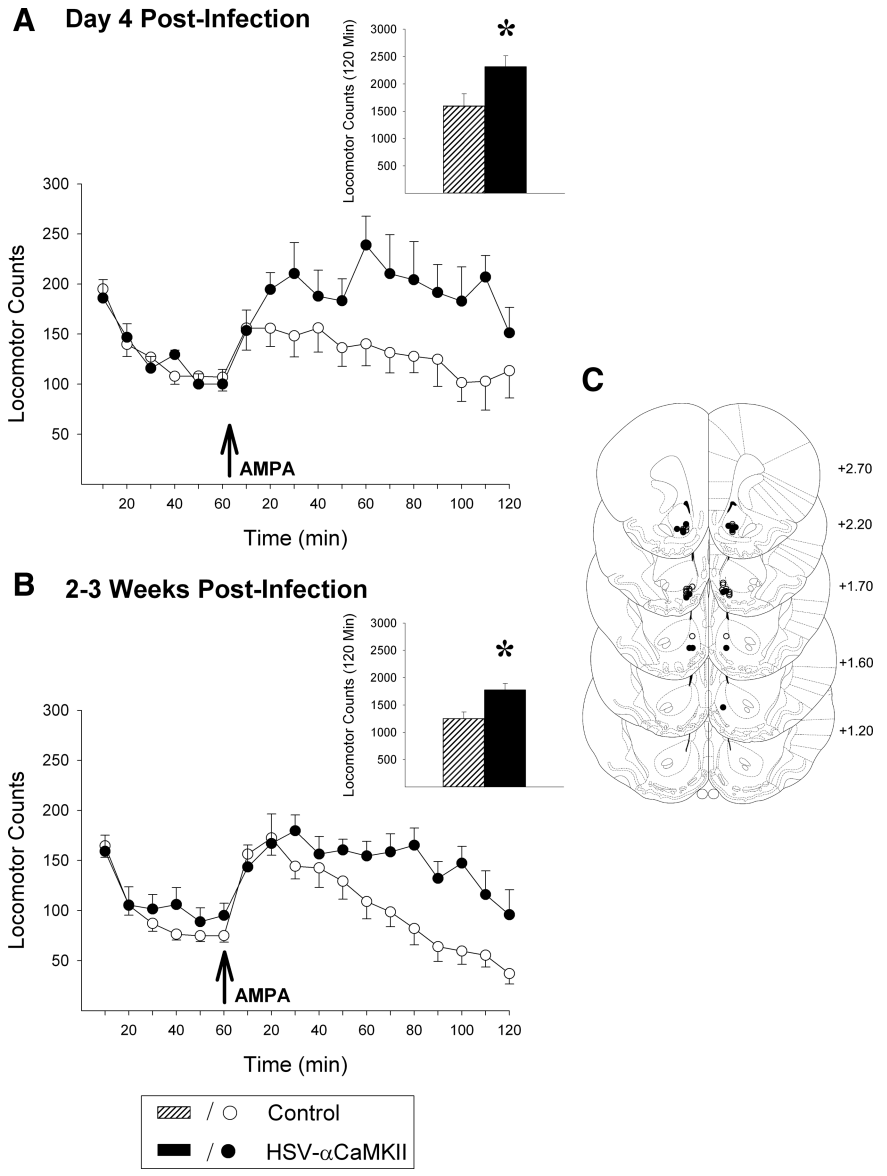

Figure 4. Transient αCaMKII overexpression in the NAcc shell enhances locomotor responding to NAcc AMPA. Data are shown as group mean locomotor counts (±SEM) before and after NAcc AMPA 4 d (***A***) or 2–3 weeks after infection (***B***). *$p < 0.05$, HSV-αCaMKII versus control. *n*/group = 7–13. The insets show group mean (+SEM) 2 h total locomotor counts obtained after AMPA for each day. ***C***, Location of the injection cannula tips for rats included in the data analyses illustrated as in Figure 3.

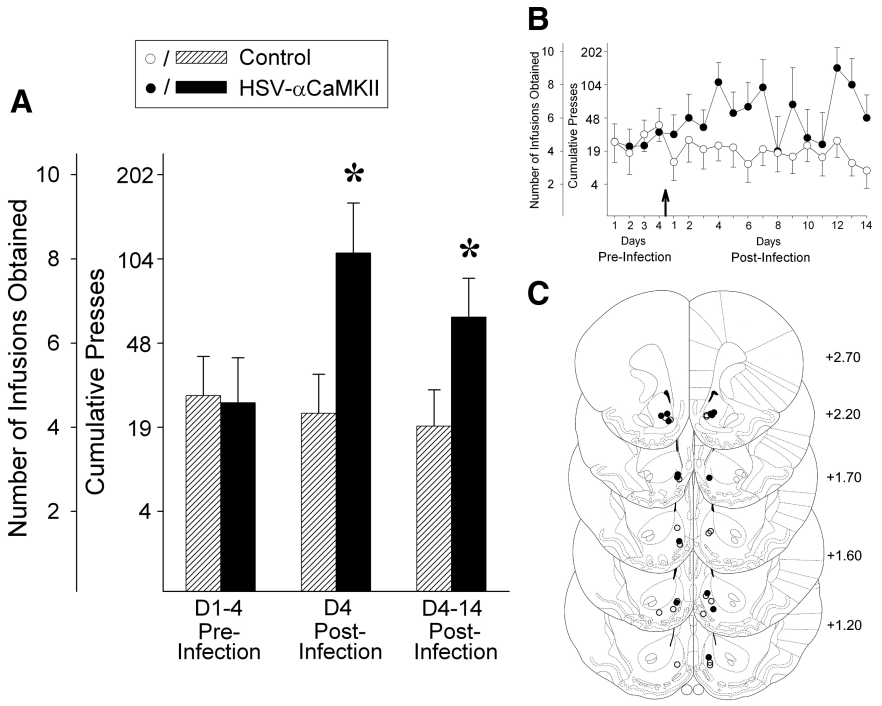

Figure 5. Transient αCaMKII overexpression in the NAcc shell enhances amphetamine self-administration. ***A***, Data are shown as group mean (+SEM) number of amphetamine infusions obtained averaged over the first 4 d before infection, on the fourth day after infection (when protein levels were maximal), and averaged over days 4–14 after infection. The cumulative number of presses required to obtain these infusions is also shown. *$p < 0.05$, significantly different from controls. *n*/group = 7–9. ***B***, Time course for amphetamine self-administration on the 4 d before infection (arrow) and the 14 d after infection. Data are shown as group mean (±SEM) number of amphetamine infusions obtained. ***C***, Location of the injection cannula tips for rats included in the data analyses illustrated as in Figure 3.

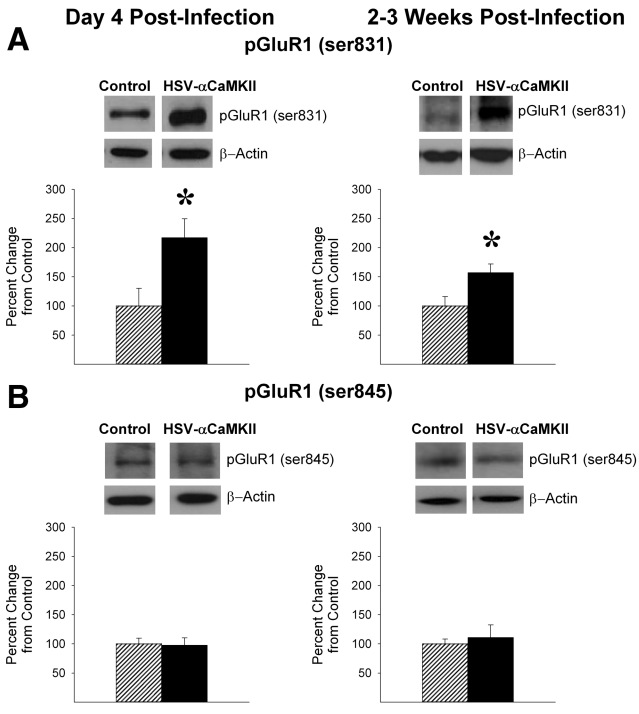

Figure 6. Transient αCaMKII overexpression increases pGluR1 in the NAcc shell. Protein levels and representative immunoblots obtained 4 d or 2–3 weeks after infection are shown as in Figure 1. Long-lasting increases in pGluR1 (Ser831) (***A***) were observed. Levels of pGluR1 (Ser845) were not significantly increased either soon or long after infection (***B***). *$p < 0.05$, HSV-αCaMKII (black bars) versus control (cross-hatched bars). *n*/group = 5–9.

reward (Kelz et al., 1999; Todtenkopf et al., 2006). In the present experiments, αCaMKII overexpression did not alter total GluR1 or GluR2 levels, indicating that alterations in AMPA receptor subunit levels were not responsible for the enhanced locomotion and amphetamine self-administration observed after NAcc shell αCaMKII overexpression. It remains to be determined whether transient viral-mediated αCaMKII overexpression also leads to increased cell surface expression of AMPA receptors.

The postphosphorylation cascades initiated by αCaMKII that lead to long-lasting functional upregulation of AMPA receptors remain to be identified. These may involve decreases in protein phosphatase activity as psychostimulant exposure has been shown to attenuate calcineurin levels and activity in striatum (Lin et al., 2002; Hu et al., 2005). Because PKC phosphorylates GluR1 (Ser831) and PKC activity is known to contribute to the expression of psychostimulant sensitization (Pierce et al., 1998; Gnegy, 2000), it is possible that amphetamine and CaMKII-mediated alterations in the activity of this enzyme also contribute. Overexpression of αCaMKII in optic tectal neurons also enhances and stabilizes synaptic strength (Wu and Cline, 1998), increases AMPA receptor-mediated transmission (Wu et al., 1996), and likely contributes to these effects by promoting the long-lasting enlargement of spines (Matsuzaki et al., 2004). As large spines are associated with increased AMPA receptor-mediated currents and have been proposed to act as stable physical traces of long-term memory (Grutzendler et al., 2002; Trachtenberg et al., 2002; Kasai et al., 2003; Matsuzaki et al., 2004), such long-lasting neuroadaptations could have resulted from transient αCaMKII overexpression and mediated the enhanced AMPA- and amphetamine-induced locomotor responding observed long after infection in the present experiments.

The transient increases in αCaMKII and the long-lasting αCaMKII-induced neuroadaptations and enhancements in behavioral responding observed in the present experiments are consistent with previous findings showing that repeated exposure to cocaine transiently increases NAcc αCaMKII levels (Boudreau et al., 2009) and that αCaMKII regulates AMPA receptor signaling in NAcc neurons (Sun et al., 2008). Recent evidence suggests that CaMKII can be recruited in these neurons by a pathway initiated by activation of D_1 DA receptors leading to phosphorylation by PKA of L-type calcium channels, an increase in inward calcium conductance, and activation of CaMKII (Surmeier et al., 1995; Hernández-López et al., 1997). Cocaine-induced reinstatement is dependent on this pathway and phosphorylation of GluR1 (Ser831) in NAcc shell neurons (Anderson et al., 2008) and D_1 DA receptor activation PKA-dependently increases GluR1 cell surface expression in primary NAcc neuron cultures (Chao et al., 2002; Mangiavacchi and Wolf, 2004; Sun et al., 2008). Consistent with these findings, the long-lasting functional upregulation of NAcc shell AMPA receptors observed after transient viral-mediated αCaMKII overexpression is dependent on

D_1 DA receptor and PKA activation (Singer et al., 2007). Considering that CaMKII also contributes to the sensitization of NAcc DA release, this enzyme may enhance behavioral output in amphetamine-exposed rats by acting presynaptically and postsynaptically in the NAcc to produce a sensitizing feedforward loop involving enhanced DA release and functional upregulation of AMPA receptors, together leading to increasing excitability in medium spiny neurons (Loweth and Vezina, 2010).

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